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DNA strand breaks produced by oxidative stress in mammalian cells exhibit 3′-phosphoglycolate termini

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ABSTRACT

In recent years two mechanisms have been proposed for the production of DNA strand breaks in cells undergoing oxidative stress: (i) DNA attack by OH radical, produced by Fenton reaction catalyzed by DNA-bound iron; and (ii) DNA attack by calcium-activated nucleases, due to the increase of cytosolic and nuclear calcium induced by oxidative stress. We set out to investigate the participation of the former mechanism by detecting and quantifying 3′-phosphoglycolate, a 3′ DNA terminus known to be formed by OH radical attack to the deoxyribose moiety, followed by sugar ring rupture and DNA strand rupture. These structures were found in DNA of monkey kidney cells exposed to hydrogen peroxide, iron nitrilotriacetate or ascorbate, all species known to favor a cellular pro-oxidant status. The method employed to measure 3′ phosphoglycolate was the ^{32}P-postlabeling assay. Repair time course experiments showed that it takes 10 h for 3′-phosphoglycolate to be removed from DNA. It was found that the DNA of both control cells and cells exposed to hydrogen peroxide had a very poor capacity of supporting *in vitro* DNA synthesis, catalyzed by DNA polymerase I. If the DNA was previously incubated with exonuclease III, an enzyme able to expose 3′-OH primers by removal of 3′-phosphoglycolate and 3′-phosphate termini the *in vitro* synthesis was substantially increased. This result shows that either of these termini are present at the break and that 3′-hydroxyl termini are virtually absent. At least 25% of the strand breaks exhibited 3′-phosphoglycolate termini as determined by the ^{32}P-postlabeling assay, but due to the characteristic of the method this percentage is likely to be higher. These results favor the hypothesis that an oxidative agent generated by Fenton reaction is responsible for DNA strand breakage in cells undergoing oxidative stress.

INTRODUCTION

Lipid peroxidation (1,2), protein oxidation (3–5) and DNA oxidative damage (6–10) are the most extensively studied molecular effects of oxidative stress. In many of these studies the participation of metal ions in the mechanism of oxidation was established (3,9,11,12). Fenton reaction, leading to the formation of hydroxyl radical (3,9,12,13) or to putative iron-oxo compounds of equivalent reactivity (12,14–16), has been proposed to intermediate most of the oxidative modifications

The Fenton reaction requires H_2O_2 and a transition metal ion in the reduced form. In biological systems Fe(II) and Cu(I) are the relevant species to be considered (17) in which case the Fenton reaction can be represented as follows: $H_2O_2 + Fe^{2+}(Cu+) \rightarrow OH^- + HO^\bullet + Fe^{3+}(Cu^{2+})$. Copper is more active than iron as a Fenton reactant (12) but iron is much more abundant in biological systems (17). The consequence is that iron is usually the redox cation that participates preponderantly in cellular Fenton chemistry involving DNA (18,19).

Several studies have indicated the participation of Fenton reaction in the production of DNA damage (9,20–25). A model was put forward according to which DNA-bound iron reacts with H_2O_2, a common product of oxygen stress and produces OH radical which can attack DNA *in situ* (21,22). More recently, ferryl compounds have also been considered to be relevant oxidants generated by Fenton reaction (12,15,16). In fact, the base modifications which originated from exposure of cells to H_2O_2 (26) are those expected by either OH radicals or ferryl compound reactions.

Another type of DNA damage produced by oxidative stress is the strand break. It has long been established that OH radicals can attack DNA to produce breaks. However, the possibility has been raised that another important mechanism of strand break production is nuclease activation by calcium, a cation whose cytosolic concentration is known to increase under oxidative stress (6,27). This mechanism would be reminiscent of apoptosis (28); in most of the works in which it was investigated the supporting evidence came from the inhibition of strand break formation by quin 2, a calcium chelator. However, more recently the possibility was put forward that the iron chelator property of quin 2 was, in fact, responsible for the inhibitory effect (19,29).

So far, the evidences favoring the DNA attack by either the Fe-driven OH radical attack or by the Ca-activated nuclease have originated from experiments with metal chelators, which are in most cases of low specificity. A direct way of examining this problem is to determine the chemical structure of the strand break moiety in DNA. Radiobiological studies have shown that the

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attack of OH radical to the deoxyribose is the major source of strand breaks and that these exhibit 5′-p with either a 3′-pg or a 3′-p terminus (30,31). The exposure of *Escherichia coli* to H_2O_2 leads to strand breaks whose properties are consistent with the presence of 3′-pg (32). However, in mammalian cells no attempt has been made to detect these structures under conditions of oxidative stress other than γ radiation exposure (33). In the present work we have shown that three different conditions of oxidative stress lead to the formation of 3′-pg termini. We were able to quantify these termini and to conclude that they contribute considerably to the total of strand breaks.

MATERIALS AND METHODS

Cells

The experiments were carried out with green monkey kidney fibroblasts (line CV1-P). They were routinely grown in Dulbecco's modified Eagles medium (DME), pH 7.0, supplemented with 10% (v/v) fetal-calf serum, 472 U penicillin/ml and 94 μg streptomycin/ml. Cells were incubated in a humidified CO_2/air atmosphere (1:19) at 37°C as previously detailed (22).

Cell treatment

Incubation of the cells with 1 mM FeNTA or 1 mM ascorbate were for 2 h in DME medium- (calcium containing PBS (137 mM NaCl, 268 mM KCl, 8.1 mM $NaPO_4$, 1.47 mM KH_2PO_4, 1 mM $CaCl_2$) PBSA, at 37°C. Stock solutions of 0.01 mM FeNTA were prepared with equimolar quantities of $FeCl_3$ and nitrilotriacetic acid, in aqueous solutions (34). The pH of these solutions was adjusted to values in the 5.0–6.5 range immediately before the cell treatment. Treatments with 1 mM H_2O_2 were for 30 min in PBSA. Concentrations of H_2O_2 solutions were determined immediately prior to the experiment (35).

DNA extraction

Cells were lysed with an extraction solution containing 0.2% SDS, 5 mM CDTA, 1 mM LiCl, 50 mM Tris–HCl, pH 8.0 and 1 M Urea. To the resulting lysate 100 μg/ml proteinase K was added and incubation proceeded overnight at 37°C. DNA was precipitated with 0.55 vol isopropanol and resuspended in a solution containing 1 mM CDTA, 10 mM Tris–HCl, pH 7.5, 50 μg/ml DNase-free RNase and incubated for 30 min at 37°C. This was followed by two extractions with equal volume of phenol/chloroform (1:1) and the DNA was precipitated with 3 vol 95% ethanol. The DNA pellet was washed by centrifugation in 70% ethanol, dried under vacuum and redissolved in water or in the buffer required for analysis. DNA concentration was measured by reading A_{260} and its purity was assessed by assuring that $A_{260}/A_{280} > 1.75$ and $A_{260}/A_{232} > 2.0$.

^{32}P-postlabeling assay

The procedure described by Weinfeld and Soderlind (36) was essentially followed; 10 μg DNA was digested overnight at 37°C with 0.08 U DNase I, 0.08 U phosphodiesterase I and 0.8 U alkaline phosphatase in 100 μl of a digestion buffer containing 4 mM $MgCl_2$ and 10 mM Tris–HCl, pH 7.5. At the end, extra quantities of 0.04 U phosphodiesterase I and 0.2 U alkaline phosphatase were added and incubation proceeded for an extra 6 h period. The enzymes were precipitated by the addition of 3 vol cold ethanol and centrifuged for 15 min at 10 000 *g*. The supernatant was dried up in a speed vac and the pellet was resuspended in 100 μl of bi-destilled water, heated at 100°C for 10 min, to inactivate residual nucleases and phosphatases and stored at –20°C. For 5′-^{32}P-labeling, 1 μg of digested DNA was incubated at 37°C with 2000 fmol [γ-^{32}P]ATP and 5 U polynucleotide kinase, in 20 μl of a buffer containing 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidin, 0.1 mM EDTA and 50 mM Tris–HCl, pH 7.5. After 40–60 min incubation, 2 μg of oligonucleotide, $(dN)_{21}$ or $(dN)_{38}$ and 2.5 U polynucleotide kinase were added and the mixture was further incubated for 30 min to allow depletion of [γ-^{32}P]ATP excess by the oligonucleotide.

The samples were diluted with 1 vol formamide buffer (90% formamide, 0.02% bromophenol blue and 0.02% xilene cyanol in 1× TBE), heated at 97°C for 3 min and immediately cooled in an ice bath. They were loaded onto a 15% polyacrylamide/ 7 M urea gel, pre-warmed at 55°C and submitted to electrophoresis at 1500–1800 V. Sequencing plates (33 × 52 cm) with 0.6 cm spacer were used in the gel setup. The samples were run up to the point when the bromophenol blue had migrated 15 cm. The gel was dried under vacuum for 2 h and the radiolabeled products were visualized by autoradiography.

Exonuclease III treatment

Ten micrograms of DNA was dissolved in 100 μl of a buffer solution containing 10 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$ and 1 mM EDTA and incubated for 5 h with 130 U exonuclease III. The DNA was precipitated with 3 vol ethanol, after adding ammonium acetate to 2.5 M. The pellet, collected by centrifugation, was washed twice by centrifugation with 70% ethanol, dried under vacuum and redissolved in the appropriate buffer for postlabeling assay or for DNA synthesis.

In vitro DNA synthesis

The assay is based on the removal of 3′-pg and 3′-p termini by exonuclease III, which generates 3′-OH primers for DNA synthesis catalyzed by DNA polymerase I (32). The reaction solution contained 50 ng of DNA, dATP, dGTP and dTTP at 20 μM each, 5 μl of [γ-^{32}P]dCTP (10 mCi/ml, ~3000 Ci/mmol), 10 U DNA polymerase I in 50 μl of 10 mM Tris–HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. At the indicated times 0.5 μl aliquots were removed from the reaction mixture and diluted in 10 μl of a solution containing 0.2 M EDTA/dioxane (1:1), loaded onto glass fiber filters (Millipore AP 15) and filtered under vacuum by adding 10 ml of 10% TCA followed by 20 ml of 95% ethanol.

Quantification of 3′-pg lesions

Two procedures were used: (i) the bands of radioactivity in the dried gel, whose position had previously been determined by autoradiography, were cut off and their Cerenkov radiation counted. This was divided by the total counting in the lane and multiplied by 2000 fmol (the original amount of ^{32}P-ATP in the phosphorylation reaction mixture loaded onto the gel). Each of these values was corrected by subtracting the corresponding values determined in a parallel experiment with control cells; alternatively (ii) the quantification of 3′-pg was carried out by densitometry of the autoradiography, using the same calculation as described

above. A Shimadzu densitometer, model CS-9000, with dual wavelength flying-spot scanner was employed for this purpose.

DNA sedimentation in alkaline sucrose gradient

The procedure was essentially that previously described (37). Briefly, the cells (pre-labeled with ^{3}H-thymidine) were washed with cold PBS and treated with a solution containing 10 mM EDTA, 100 mM NaCl and 1% Triton X-100, for 1 min at 37°C. This dissolves out the cytoplasm, leaving the nuclei attached to the bottom of the plate. The nuclei were lysed by incubation with a solution containing 0.3 M NaOH, 1 M NaCl and 0.01 M EDTA, for 1 h at 37°C; 0.2 ml of the resulting solution was layered on the top of 4.5 ml alkaline sucrose gradients (5–20% sucrose, w/v) in 0.3 M NaOH, 1.0 M NaCl and 0.01 M EDTA and centrifuged at 25 000 r.p.m. for 2 h at 20°C in a SW 50.1 rotor of a Sorvall Combe ultracentrifuge. The radioactivity of the fractions collected from the bottom of the tube was measured as described elsewhere (38). The molecular weight of DNA for each fraction was determined by Studier's expression (39), using T7 DNA as standard. The number average molecular weights (M_n) were obtained according to the expression $M_n = \Sigma r_i/\Sigma(r_i/M_i)$ where r_i and M_i are radioactivity and molecular weight of DNA in fraction i respectively. Sedimentation profiles are not presented in this report but typical examples have been previously shown (37). The number of breaks was calculated by the formula (M_n control/M_n treated) – 1. The values were converted per 10^5 kDa.

Materials

[γ-^{32}P]ATP (10 mCi/ml, 5 Ci/mol) and [α-^{32}P]dCTP (10 mCi/ml, ~3000 Ci/mmol) were from Amersham. Oligonucleotides were synthesized by National Biosciences, Inc. The other biochemical reagents were obtained from Sigma.

Enzymes

DNase I (Sigma). One Kunitz unit produces an increase in A_{260} of 0.001/min/ml, at pH 5.0, 25°C, using DNA as substrate, at 4.2 mM Mg^{2+}. Calf intestinal alkaline phosphatase (Gibco-BRL). One unit hydrolyzes 1 µmol of *p*-nitrophenyl phosphate in 1 min at 37°C. Phosphodiesterase I from *Crotalus atrox* venom (Sigma, type VII). One unit hydrolyzes 1 µmol of bis (*p*-nitrophenyl) phosphate per min at pH 8.8, 37°C. Polynucleotide kinase (Pharmacia). One unit catalyzes the transfer of 1 nmol of phosphate from ATP to polynucleotide in 30 min at 37°C. DNA polymerase I (Pharmacia). One unit catalyzes the incorporation of 10 nmol of deoxynucleotides into acid-insoluble product in 30 min at 37°C, with poly (dAdT)·poly (dAdT) as the template. Exonuclease III (Gibco-BRL). One unit produces 1 nmol of acid-soluble nucleotide from sonicated DNA in 30 min at 37°C.

Results

Presence of 3′-pg termini in DNA from cells submitted to oxidative stress

To establish the conditions of 3′-pg termini detection by postlabeling assay, calf thymus DNA, $(dA)_5$, $(dC)_5$, $(dG)_5$ and $(dT)_5$ (all at 150 µg/ml) were irradiated with 150 Gy of γ-rays and processed as described in Materials and Methods. The resulting autoradiogram of the electrophoresis is shown in Figure 1. The indicated bands were assigned to deoxynucleoside 5′-phosphate,

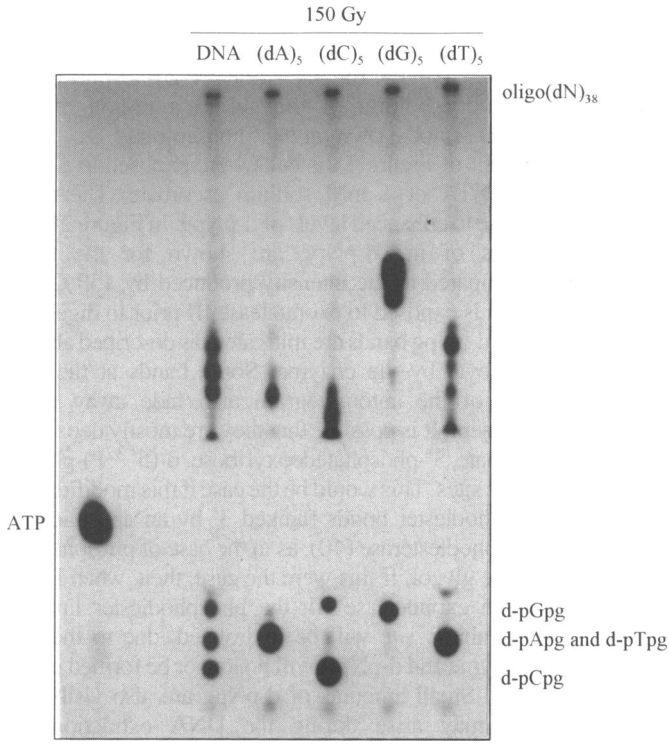

Figure 1. PAGE migration patterns of nucleotides bearing 3′-phosphoglycolate. Calf thymus DNA and oligos $(dA)_5$, $(dC)_5$, $(dG)_5$ and $(dT)_5$ were dissolved in water (150 mg/ml), irradiated with 150 Gy and submitted to ^{32}P-postlabeling assay. Oligo$(dN)_{38}$ was used to deplete the excess [γ-^{32}P].

3′-phosphoglycolate (d-pNpg) on the basis of (i) pattern of migration: the position, ahead of ATP and the relative mobilities of the four authentic d-pNpg (36) are equivalent to the ones shown in Figure 1, obtained under similar experimental conditions; (ii) mitigation of the bands when DNA was treated with exonuclease III prior to the postlabeling assay (see below). This enzyme is known to remove 3′-pg termini from DNA (32). Upon further enzyme digestion with DNase I, phosphodiesterase I and alkaline phosphatase, the resulting mononucleosides no longer bear the 3′-pg terminus and cannot be phosphorylated by polynucleotide kinase.

On the $d(C)_5$ lane a small spot above the d-pCpg band has been consistently observed, the nature of which is not known. We suspect that under the harsh irradiation conditions part of the dC is deaminated to dU, in which case the minor band should correspond to d-pUpg.

At the low migration region of the gel several bands are present, with different mobilities for the four $(dN)_5$. It is known that under aerobic conditions γ-irradiation produces a large number of DNA lesions. No effort was made to characterize these lesions in the present work. On the top of the autoradiogram is the oligodeoxynucleotide added at the end of the postlabeling assay to deplete excess of [γ-^{32}P]ATP. Its labeling intensity varied from one experiment to another, depending on the amount of [γ-^{32}P]ATP left after the postlabeling assay.

An unidentified band is visible migrating slightly faster than the d-pNpg. It is equally present in all DNA samples irrespective of whether the cells have been treated or not. Therefore they should

not constitute a DNA modification. It is only labeled significantly in experiments in which the postlabeling time was longer than 30 min, as in Figures 2, 4 and 5. Finally, some ^{32}P-*ortho*-phosphate migrates at the very bottom of the gel in a region of the autoradiogram that is not shown on the photographs.

In the experiment of Figure 2 the cells were exposed to 1 mM H_2O_2, 1 mM FeNTA or 1 mM sodium ascorbate. The three treatments gave rise to enhanced levels of d-pNpg. In Figure 2B the relative intensities of the d-pNpg are shown for the three treatments, as compared to the intensity produced by 150 Gy of γ-rays. If the DNA is exposed to exonuclease III prior to digestion with enzymes, the d-pNpg bands are mitigated, as described above, due to 3′-pg removal by the enzyme. Some bands at the low migration region of the autoradiogram also fade away upon exonuclease treatment. It is possible that they are mostly deoxynucleoside 5′-phosphate, 3′-phosphatedeoxyribose, d-(5′ ^{32}P)-pNpR, arising from abasic sites. This would be the case if this modification renders the phosphodiester bonds flanked 3′ by an apurinic site refractory to phosphodiesterase (40), as in the case of phosphoglycolate and thymine glycol. If this were the case, then, when DNA is pre-treated with exonuclease III, the phosphodiester linkage flanked 3′ by an abasic site will be hydrolized, due to the AP activity of this enzyme and d-pNpR will no longer be formed in the postlabeling assay. Small amounts of d-pNpg are also visible in control cells. It may arise during the DNA extraction or, alternatively, may be present in DNA under normal cellular metabolic conditions. In this respect, 8-hydroxy-2′-deoxyguanosine has been found in cells of untreated animals (41).

Exonuclease III is capable of removing 3′-pg and 3′-p from DNA 3′ termini, producing 3′ OH termini which is primer for DNA synthesis catalyzed by DNA polymerase I (32). In the experiment of Figure 3, DNA from control cells or from cells treated with H_2O_2 are poor substrates for deoxynucleoside triphosphate incorporation by DNA polymerase I. However, if these DNA's are previously incubated with exonuclease III, they become effective primers for DNA polymerase I. As expected, the DNA from cells treated with H_2O_2 provided more 3′-OH primers for DNA synthesis after exonuclease treatment than the control cells. These results indicate that DNA from both control cells and H_2O_2-treated cells have 3′ termini bearing pg and/or phosphate and very few, if any, 3′ OH termini.

A dose–response curve for formation of d-pNpg is shown in Figure 4A. Increasing H_2O_2 concentrations lead to progressively more intense d-pNpg bands. To quantify these damages, the band intensities were measured by a densitometer or the bands were cut out from the gels and their radioactivity was determined (Fig. 4B). It can be seen that above 1 mM the trend is the amount of d-pNpg levels off as a function of the H_2O_2 concentration. This saturation effect had been previously observed when measuring DNA strand breaks as a function of H_2O_2 dose and was ascribed to a limiting effect imposed by the number of iron ions bound to DNA (37). It is also possible that distinct types of oxidants are formed by iron-mediated Fenton reactions in the presence of DNA (16). These oxidants would be formed at different H_2O_2 concentration ranges and might depend on different types of interactions between Fe(II) and DNA. In this case, one might consider that only one of them would be responsible for production of 3′-pg.

The 3′-pg termini are repaired as can be seen in the time course experiment of Figure 5. It takes ~10 h for the level of d-pNpg to attain the control level. Apparently, the control level of 3′-pg

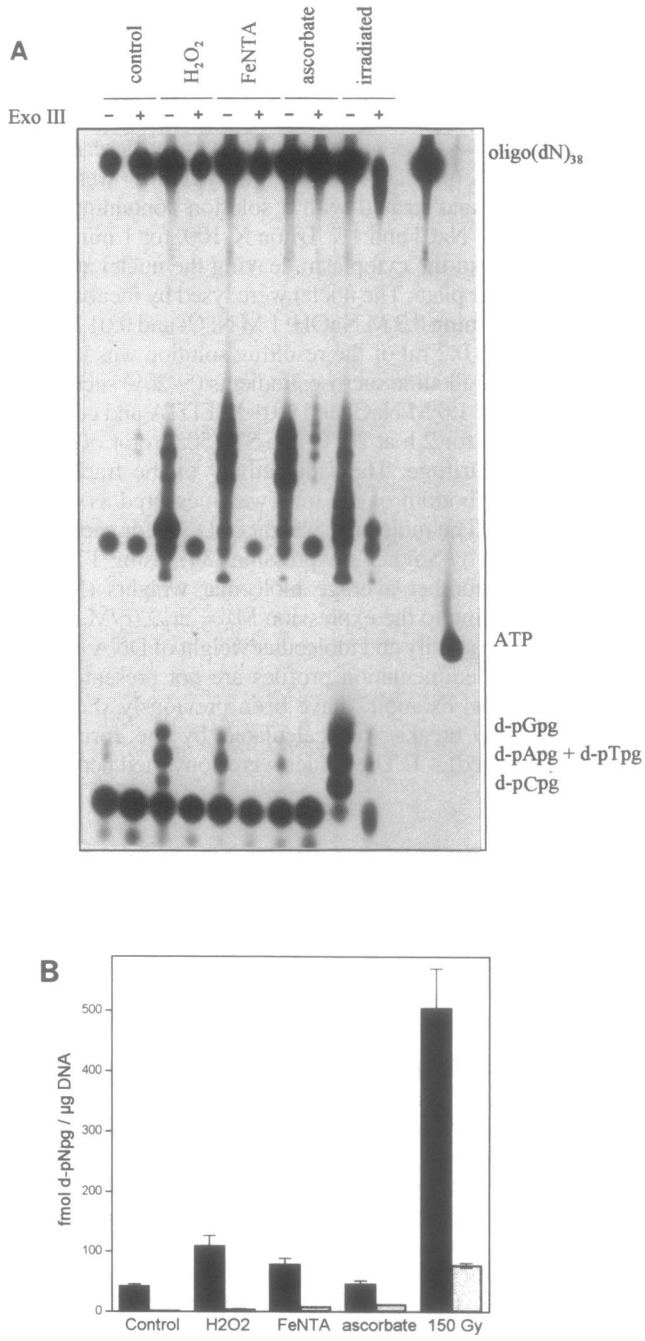

Figure 2. Mitigation of 3′-phosphoglycolate termini by exonuclease III treatment of DNA from CV1-P cells exposed to oxidative stress. Exposures were at 37°C for 2 h in DME with 1 mM FeNTA or 1 mM ascorbate and for 30 min in PBSA with H_2O_2. Incubation of DNA with exonuclease III was for 5 h before postlabeling. (**A**) Page autoradiogram. The control lanes refer to DNA extracted from untreated cells. The 150 Gy irradiated DNA was run in parallel to provide a previously established pattern of 3′-pg nucleotide bands (see Fig. 1). Oligo(dN)$_{38}$ was used to consume the bulk of the excess ATP. (**B**) Quantification of the 3′-phosphoglycolate bands. Black bars correspond to untreated DNA. Grey bars correspond to DNA incubated with exonuclease III before postlabeling. The error bars correspond to the deviation of the mean for two independent experiments, one made by densitometry of the bands in the autoradiogram shown in A and the other one corresponding to Cerenkov counting of a separate autoradiogram, not shown.

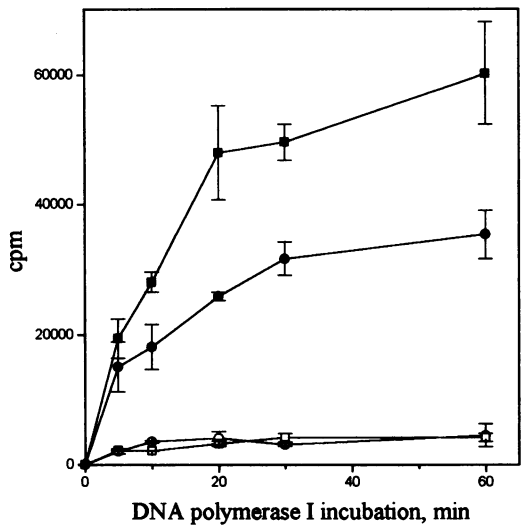

Figure 3. *In vitro* activation of DNA synthesis. DNA extracted from untreated cells (○) or from cells exposed for 30 min to 1 mM H_2O_2 in PBSA (□) were incubated with exonuclease III (closed symbols), prior to [α-^{32}P]dCTP incorporation with DNA polymerase I. The total radioactivity for each DNA synthesis reaction was 3×10^5 c.p.m. Each point represents the mean of two radioactivity countings in 0.25 µl aliquots. The initial volume of the reactional mixture was 50 ml and contained 50 ng of DNA.

termini is maintained because an equilibrium between the rates of formation and of repair of these lesions is attained. In the experiments of Figures 4 and 5 and in other experiments not shown it is clear that the d-pCpg band is much fainter than those of the other 3′-pg nucleotides. This is an intriguing finding that may be correlated to the site-specific nature of the Fenton reaction responsible for the DNA ssb. However, it is also possible that repair of d-pCpg is faster than those of the other d-pNpg.

We attempted to determine which proportion of the DNA ssb exhibited 3′-pg terminus by comparing the number of these two DNA modifications. For this purpose, part of the cells treated with H_2O_2 were lysed, for DNA ultracentrifugation in alkaline sucrose gradients, and the remaining cells provided the DNA for the postlabeling assay. Table 1 shows that 1 mM H_2O_2 produces an average of 29.6 ssb/10^5 kDa and 7.9 d-pNpg/10^5 kDa. Therefore an average of 25% of the ssb features a 3′-pg.

DISCUSSION

The recent controversy on the mechanism(s) involved in the production of DNA ssb by oxidative stress, whether Ca^{2+}-activated nucleases or Fe-induced OH radical formation (27,42) requires investigation in different systems in order to assess their relative importance. The latter mechanism implies that OH radical (9) or an iron-oxo oxidant of equivalent reactivity (15,16,43) is formed in the vicinities of the DNA. In this context, the involvement of either species in DNA damage has already been corroborated by detection of H_2O_2-induced base oxidation in experiments with cultured cells (26). However, there remains the question as to whether Ca^{2+}-activated nuclease would not be more relevant in the case of DNA strand breaks.

So far, the support for the role of Ca^{2+}-activated nuclease derives from experiments in which the Ca^{2+} chelator quin 2 was shown to prevent DNA ssb by oxidative stress (27,44,45). The possibility that quin 2 can act as an iron chelator has been considered, but contrarily to 1,10-phenanthroline (9) and to desferral (11), the Fe–quin 2 complex does not prevent Fe-mediated OH radical formation (19,27,42). Therefore, it was concluded that in Chinese hamster cells exposed to H_2O_2, quin 2 prevented nuclease Ca^{2+} activation and the consequent DNA ssb (26). Nevertheless, the use of quin 2 as a probe for participation of Ca^{2+} in DNA damage by oxidative species has been recently challenged based on two observations: (i) the Fe(iii)–quin 2 complex is not reduced by O_2^- and this might be required for OH radical formation (29); and (ii) quin 2 inhibits DNA oxidation by H_2O_2 *in vitro*, which was attributed to the removal of DNA-bound iron by quin 2, causing in this manner a mitigation of site-specific Fenton reaction (19).

We decided to examine this question by investigating the formation of 3′-pg termini, a structure known to be formed at the sites of DNA chain interruption and that originates from OH radical attack to the deoxyribose moiety of γ-irradiated DNA (46,47). Under three conditions of oxidative stress, produced respectively by exposure of cells to H_2O_2, FeNTA and ascorbate, d-pNpg were clearly detected by using a postlabeling assay (36). These structures were not observed when the DNA was previously digested with *E.coli* exonuclease III. This is in agreement with the fact that this enzyme is capable of removing the 3′-phosphoglycolate moiety to generate a 3′-OH terminus (48).

The removal of 3′-pg is important to provide a 3′-OH primer for DNA synthesis. The exonuclease III treatment of DNA from H_2O_2-treated cells, and to a minor extent from control cells, provided the 3′-OH primers to sustain dNMP incorporation from dNTP (Fig. 3). It is to be noticed that without exonuclease III digestion these DNA's were very poor primers for DNA polymerase I, indicating that most of the single strand interruptions bear 3′-pg or 3′-p termini.

Table 1. Comparison between the frequencies of DNA ssb and of d-pNpg in H_2O_2-treated cells

H_2O_2 (mM)	$M_n \times 10^5$ kDa	nu. ssb/10^5 kDa	fmol d-pNpg/µg DNA	nu. d-pNpg/10^5 kDa	pg/ssb (%)
1.0	3.0	26.3	64 ± 3	6.4	24.3
1.0	2.4	32.9	94 ± 14	9.4	28.6

The data are from two independent experiments in which the cells were exposed to 1 mM H_2O_2. The number average molecular weight (M_n) for the DNA from control cells in the two experiments were 14.3×10^5 and 11.3×10^5 kDa respectively. The d-pNpg values in the fourth column were determined by subtracting the corresponding values of control DNA (61 ± 20 and 32 ± 1 fmol/µg DNA respectively) from the values determined for DNA from H_2O_2-treated cells. The mean and the deviation are for independent Cherenkov counting in two separate electrophoresis for each experiment.

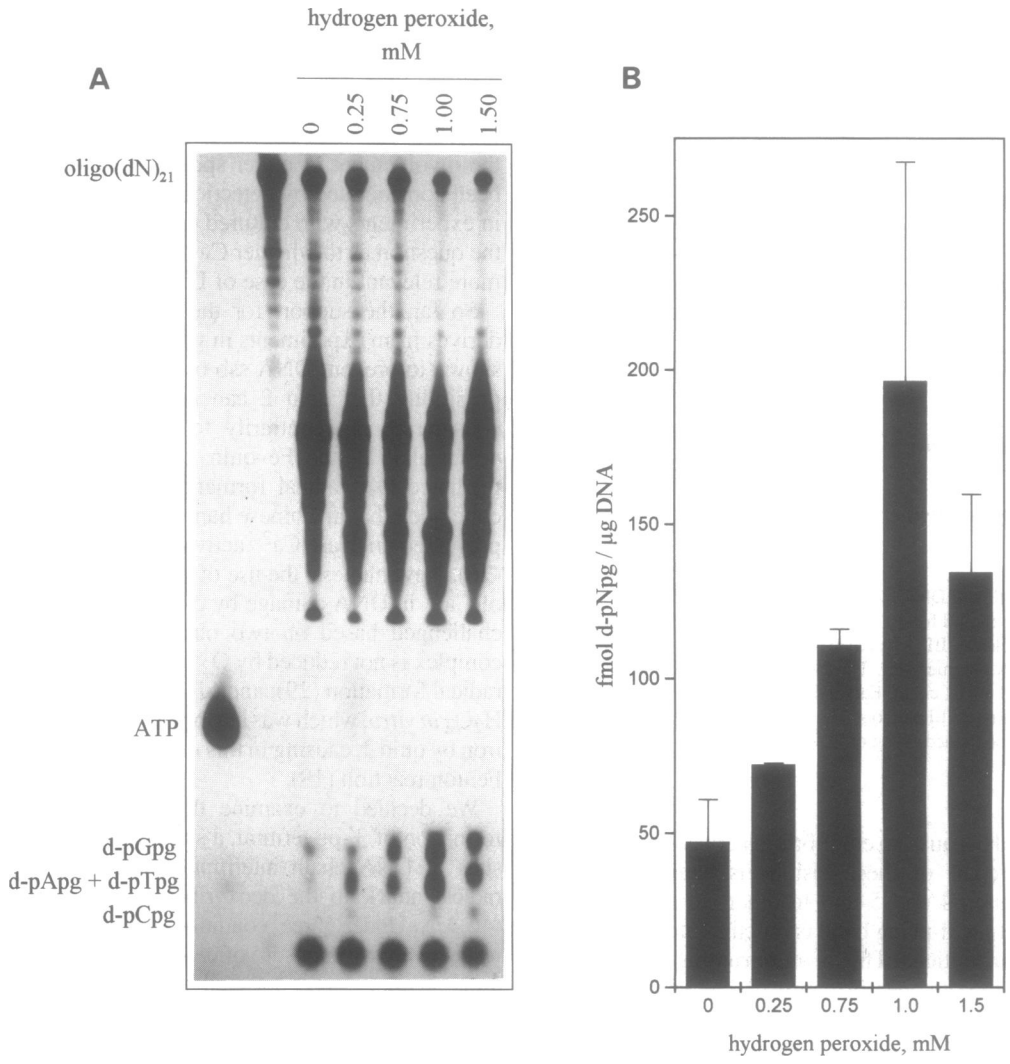

Figure 4. Dose–response for formation of 3′-phosphoglycolate termini in DNA from H_2O_2-treated cells. The DNA was extracted from cells exposed to 0–1, 5 mM H_2O_2 and processed by the ^{32}P-postlabeling assay. (**A**) PAGE autoradiogram. (**B**) Quantification of the 3′-phosphoglycolate bands. The error bars correspond to the deviation for two independent determinations, one made by Cerenkov counting of the bands in the autoradiogram shown in A and the other one made by densitometry in a separate electrophoresis, not shown.

The d-pNpg produced by 1 mM H_2O_2 treatment were repaired by relatively slow kinetics, requiring 10 h to achieve the control values. It is interesting that the time course of ssb repair, measured in parallel (not shown), was even slower than the repair of d-pNpg, indicating that the latter process does not constitute the rate limiting step for strand break repair. Several activities of 3′-pg removal in eukaryotic cells have been reported aim (48,49). In one case the mechanism of 3′-phosphoglicolate removal was investigated with a purified recombinant abasic endonuclease. It was shown that the enzyme releases phosphoglycolate and phosphate from 3′-pg and 3′-p respectively (49).

The most important piece of information in the present work refers to the chemical nature of the 3′ terminus at DNA chain interruptions, caused by oxidative stress in cultured mammalian cells. Exposure of bacteria to H_2O_2 produces blocked 3′-termini that can be removed by exonuclease III providing primers for DNA polymerase I (32). These results are consistent with the generation of 3′-pg and 3′-p by H_2O_2 treatment. The situation in mammalian cells however is more complex since, as discussed above, activated nucleases can come into play under oxidative stress . In an attempt to quantify 3′-pg termini in cells exposed to H_2O_2 we compared its number, determined by the postlabeling assay, with the number of ssb, measured by sedimentation assay. The results show that ~25% of the ssb have 3′-pg termini. However, it should not be concluded that the remaining interruptions are caused by Ca^{2+}-activated nucleases since: (i) OH radical induces both 3′-pg and 3′-p termini in variable proportions, depending of the nature of the oxidative stress (30,36,50,51); (ii) the experiment of Figure 3 indicates that very few breaks bear a 3′OH terminal. To our knowledge the mechanism of phosphodiester hydrolysis by Ca^{2+}-activated nucleases has not been defined. If it produces 3′-p termini then part of these will be produced by nuclease action and part by the OH radical attack to the deoxyribose moiety and their proportion

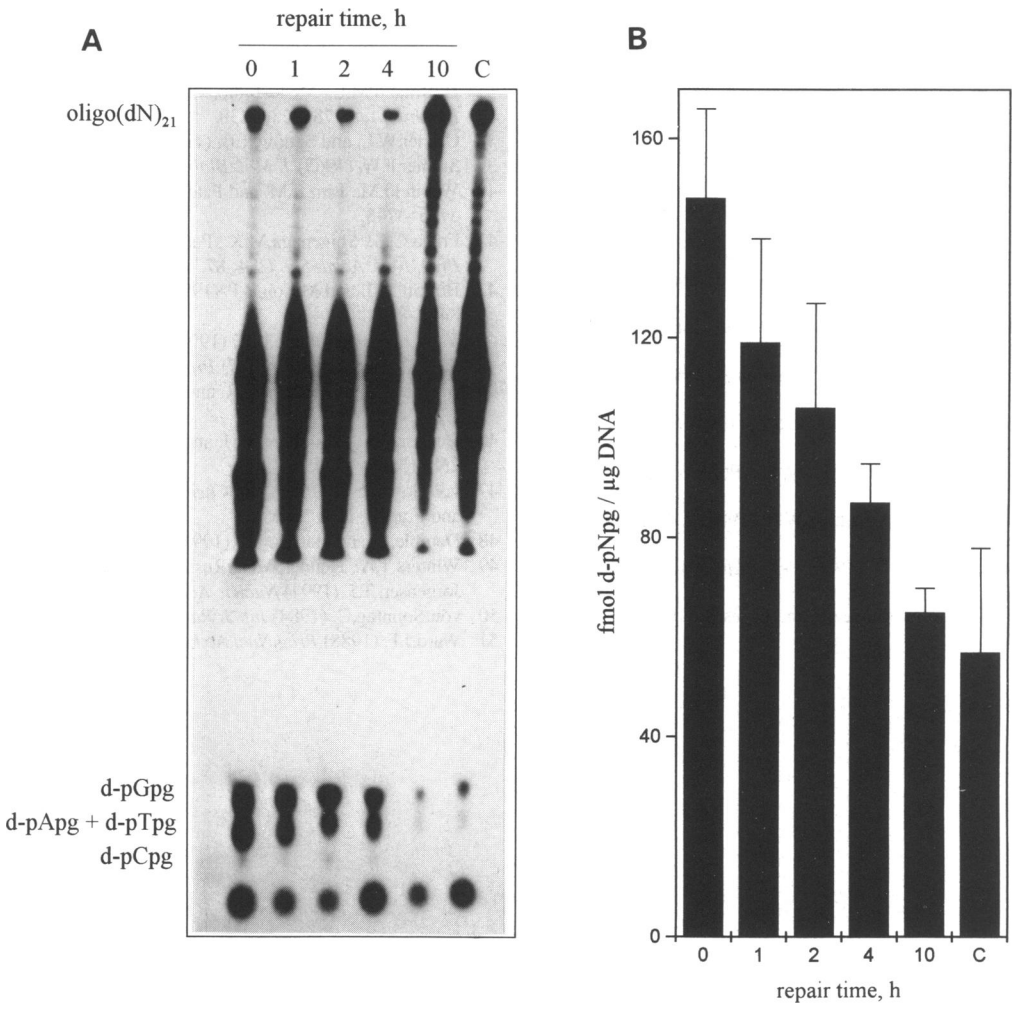

Figure 5. Repair time course of 3′-phosphoglycolate produced by H_2O_2 in CV1-P cells. The DNA was extracted from cells incubated for 1–10 h in normal medium after 1 mM H_2O_2 treatment. The control lane corresponds to DNA from untreated cells. (**A**) PAGE autoradiogram. (**B**) Quantification of the 3′-phosphoglycolate bands. The error bars have the same explanation as in Figure 4 legend.

can not be deduced from the present experiment. If it produces 3′-OH termini then its activity in these cells is negligible. In this case most of the remaining 3′ termini would be constituted of 3′-p, arising from OH radical attack to the deoxyribose moiety.

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